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MALAT-1 interacts with hnRNP C in cell cycle regulation

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ABSTRACT

As a conserved non-coding RNA gene, transcripts of MALAT-1 localize predominately in the nucleus. However in G2/M cell cycle phase, MALAT-1 transcripts were surprisingly found to translocate from the nucleus into the cytoplasm. Investigation also found that in this process MALAT-1 interacts with an abundant nuclear factor, hnRNP C protein. Using a loss-of-function assay, we found that down-regulation of MALAT-1 expression compromised the cytoplasmic translocation of hnRNP C in the G2/M phase and resulted in G2/M arrest. In addition to characterize the physiological interaction between MALAT-1 and hnRNP C, our study also highlights the role of MALAT-1 in cell cycle regulation.

1. Introduction

Recent genome-wide studies revealed that most of the human and mouse genomes are transcribed to yield thousands of long non-coding RNAs with little or no protein-coding potential [1,2]. Long non-coding RNAs are mRNA-like RNA transcripts ranging from 200 nt to >100 knt in size. Even though many of them exhibit cell-type-specific expression profiles [3] and have been implicated in many essential cellular activities including proliferation and differentiation [4], mechanistic evidence supporting their physiological functions is still absent for them.

Among these, MALAT-1 (metastasis-associated lung adenocarcinoma transcript 1) is a long non-coding RNA species distributed mainly in the nucleus. It was first found in non-small cell lung tumors and then in many cancer types [5–7]. In addition to cancer tissues, high expression levels of MALAT-1 have been reported in a number of normal tissues, including brain [8]. The transcripts of MALAT-1 are localized mainly in nuclear speckles and function in pre-mRNA splicing by modulating SR splicing factor [9]. In cultured hippocampal neurons, MALAT-1 modulates synaptogenesis [8]. Down-regulating its expression decreases synaptic density and up-regulating its expression increases cell-autonomous synaptogenesis [8]. More recently, it was reported that MALAT-1 interacts with unmethylated polycomb 2 protein to activate the growth-control program. Silencing MALAT-1 expression in HeLa cells causes the loss of serum-stimulated cell proliferation and profound G1/S arrest [10]. However, by modulating the level of pre-mRNA splicing factor and the phosphorylation status of several SR family splicing factors, Tripathi and colleagues found G2/M arrest and increased cell death in MALAT1-depleted HeLa cells [9]. This recent evidence suggests that MALAT-1 might play a role in cell cycle regulation.

Heterogeneous nuclear ribonucleoprotein C (hnRNP C) is one of the most abundant nuclear proteins essential for mRNA metabolism, cell proliferation and differentiation [11,12]. Previous studies showed that HnRNP C translocates from the nucleus to the cytoplasm in the G2/M phase to increase the IRES-dependent translation of c-myc [13,14]. Supporting these findings, another study also reported that hnRNP C regulates IRES-mediated translation in G2/M phase and found that this translocation is indispensable for cells to progress through the G2/M phase [15]. Even so, it is not clear how hnRNP C protein translocates from the nucleus to the cytoplasm to function in regulating mitosis.

To elucidate the potential activities of MALAT-1 in cell cycle regulation, we first examined the distribution of MALAT-1 transcripts in different phases of the cycle, and surprisingly found that the transcripts partially translocated from the nucleus to the cytoplasm in G2/M phase. Further investigation showed that MALAT-1 interacted with hnRNP C in the cytoplasm, and this was of critical importance to the cytoplasmic translocation of hnRNP C. Down-regulating the expression of MALAT-1 not only compromised the translocation of hnRNP C in G2/M phase, but also led to G2/M arrest in cultured cells. This evidence therefore integrates long non-coding MALAT-1 into an established cell cycling network

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centered on hnRNP C protein, and further demonstrates the essential role of MALAT-1 in cell cycle regulation.

2. Materials and methods

2.1. Cell culture and synchronization

HepG2 and HeLa cell lines were purchased from ATCC and grown in DMEM supplemented with 10% fetal calf serum and 1% penicillin/streptomycin at 37 °C under 5% CO_2. For cell-cycle synchronization, the procedure described by Yang et al. [16] was used. A double thymidine block was performed to obtain cell populations in S phase, and Thymidine-nocodazole block was performed to obtain cell populations in G1 and M phase. The cells were synchronized at the G2/M boundary by a double-thymidine block, released for 6 h, and then exposed to nocodazole (100 ng/ml).

2.2. Immunofluorescence staining

Immunolocalization of proteins was performed as described previously [17]. Briefly, cells were fixed in 2% formaldehyde for 15 min at room temperature and permeabilized in 0.5% Triton X-100/PBS for 10 min on ice. Cells were incubated with primary and secondary antibodies in a humidified chamber, and finally stained with DAPI and mounted using PPD. The immunofluorescence was visualized using a fluorescence microscope.

2.3. RNA and protein isolation

Isolation of cytoplasmic or nuclear RNAs and proteins was performed using the PARIS™ kit (Ambion) and the NE-PER kit (Thermo). After RNA isolation, cDNA was synthesized with SuperScript II (Invitrogen) and qPCR assays were performed using SYBR Green A PCR mix. Relative expression values were calculated (ΔΔCT method), using GAPDH or 18s RNA as an internal control.

For protein fractionation, the cells were harvested by trypsin–EDTA digestion and centrifugation. Cytoplasmic and nuclear protein fractions were then obtained using NE-PER protein extraction kit (Thermo Scientific), following the manufacturer's recommendations.

2.4. Gene cloning and RNA pull-down

Fragments of MALAT-1 were sub-cloned from the full-length cDNA sequence. For RNA pull-down, MALAT-1 fragments were labeled with biotin by T7 RNA polymerase-mediated transcription. Five picomoles of biotinylated RNA was incubated with pre-blocked Streptavidin agarose beads (Invitrogen) at 4 °C for 5 h, followed by extensive washes to obtain biotinylated RNA-conjugated agarose beads. Then, 200 μg of splicing-competent HepG2 cell extract was added and incubated for 1 h at 30 °C, with slow rotation. To extract MALAT-1-interacting proteins, the beads were washed and boiled in SDS buffer, and the retrieved protein was analyzed by 20% SDS–PAGE and silver staining.

2.5. Protein characterization by MS

Protein bands were digested in gel by trypsin. Extracted peptides were loaded onto a C18 reverse-phase column with a pulled tip (100 μm ID; 8 cm in length), packed with 3-μm, 125-Å Aqua C18 resin (Phenomenex). LC–MS/MS analysis was performed over a 66-min run on an LTQ-Orbitrap mass spectrometer (ThermoFisher Scientific) connected to an Agilent 1200 quaternary HPLC Pump.

2.6. RNA Immunoprecipitation

A modified Nature protocol [18] was performed in RIP. One milliliter of Trizol (Invitrogen) was added to the beads to extract protein-interacting RNA, which was then treated with RNase-free DNase I (NEB) and measured by RT-PCR assay.

2.7. siRNA transfection and flow cytometry assay

Chemically synthesized siRNAs (Supplementary Table 2) were transfected into cultured cells using Lipofectamine 2000 (Invitrogen). Harvest the cells after 48 h and detect the gene silencing effect.

For the flow cytometry assay, the procedure described by Yang et al. [16]. Cell cycle profiles were assessed by a BD Biosciences FACScan with MODFIT software. At least 10000 cells in each sample were analyzed to obtain a measurable signal, using the same instrument setting.

2.8. Rescue assay

HnRNP C cDNA was PCR amplified from total RNA, digested with Xhol and Hindlll, and cloned into pcDNA3.1 expression vector. The fusion or control vectors were then transfected into Hela cells with MALAT-1-targeting siRNA. FACS assays were performed 48 h after transfection.

2.9. Cell proliferation assay

Asynchronously growing Hela cells were transfected with MALAT-1-targeting siRNAs and an irrelevant siRNA control. After transfection, the numbers of the cells was counted at 0 h, 24 h, 48 h, 72 h and 96 h. Then, a growth curve was plotted to examine the effects of the siRNAs on cell proliferation. The experiments were repeated for three times.

3. Results

3.1. MALAT-1 translocates from the nucleus to the cytoplasm in G2/M phase

MALAT-1 transcripts are localized predominantly in nuclear speckles. However, when we profiled their distribution throughout the cell cycle, we were surprised to find that they partially translocated from the nucleus to the cytoplasm in the G2/M phase in synchronized HeLa cells, which was in sharp contrast to the unsynchronized cell populations (Fig. 1A). To confirm this observation, the change of cytoplasmic levels of MALAT-1 in G2/M was determined using quantitative RT-PCR. This revealed a more than 4-fold increase of cytoplasmic MALAT-1 in G2/M-synchronized cells, in contrast to the comparable total MALAT-1 levels in synchronized and unsynchronized cell populations (Fig. 1B). Additionally, analysis of the nuclear/cytoplasmic distribution of MALAT-1 during the cell cycle presented a distinct profile, showing a profound enrichment in cytoplasm in G2/M phase (Supplementary Fig. 1).

The synchronization was performed by thymidine-nocodazole block, and the separation of nuclear and cytoplasmic fractions were made using a commercial kit. To confirm the synchronization of the cells, FACS assays were performed to show that more than 90% of the cells are in G2/M cell cycle phase; and cyclin B1, a characterized G2/M marker was shown highly expressed in the synchronized cells (Fig. 1C). To further confirm the integrity of the nuclear membranes, immuno-staining of a nuclear membrane

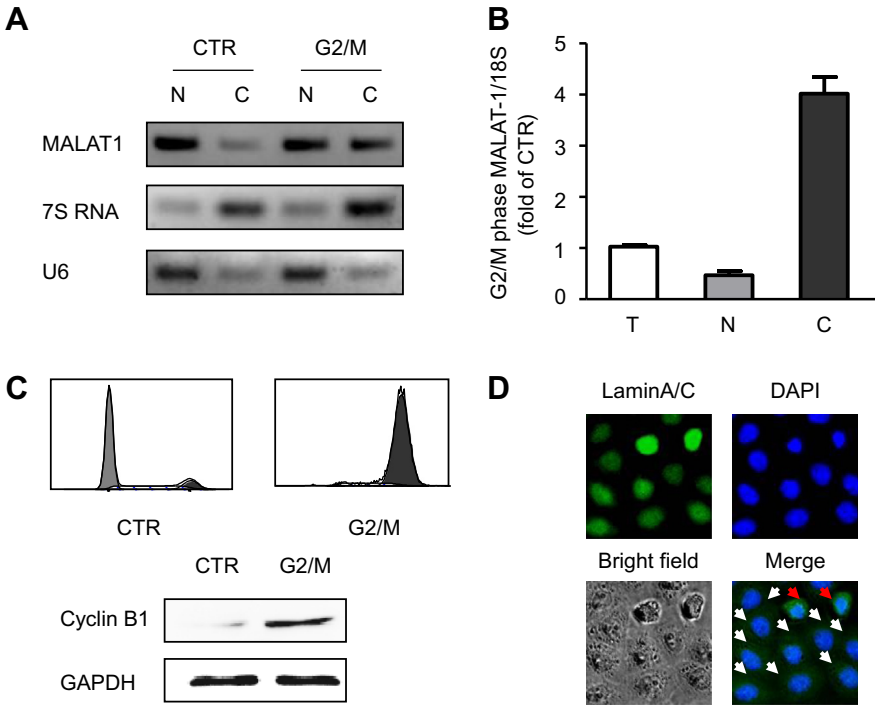

Fig. 1. Translocation of MALAT-1 RNAs from nucleus to cytoplasm in G2/M cell cycle phase. (A) The sub-cellular localization of MALAT-1 transcripts is detected at G2/M phase in Hela cells. Cytoplasmic and nuclear RNAs are isolated from unsynchronized (CTR) and synchronized (G2/M) cells, and their levels are analyzed by RT-PCR. 7S RNA and U6 RNA are included as cytoplasmic and nuclear markers. N, nuclear RNA; C, cytoplasmic RNA. (B) The levels of MALAT-1 are quantified by qRT-PCR in RNA samples extracted from synchronized (G2/M) and unsynchronized (CTR) Hela cells. 18S rRNA is included as an internal control and the results are normalized to CTR and presented as mean ± S.D. (n = 3). T, total RNA; N, nuclear RNA; C, cytoplasmic RNA. (C) Up panel: HeLa cells are synchronized in G2/M phase by thymidine-nocodazole treatment, and the cell cycle profiles are determined by propidium iodide (PI) staining and flow cytometry assay. Low panel: the levels of Cyclin B1 and GAPDH are determined in cell extracts from unsynchronized (CTR) and synchronized (G2/M) cell populations, using Western blot assay. (D) Cells in G2/M phase were immunostained for nuclear envelope marker LaminA/C (green fluorescence), using an Alexa-conjugated secondary antibody. The nuclei were counterstained with DAPI (blue fluorescence). Red arrow, M-phase cells; white arrow, G2 phase cells.

marker LaminA/C was also performed (Fig. 1D), revealed intact nuclear membrane for majority of the isolated cells. The successful separation of the nuclear and cytoplasm components was demonstrated by RT-PCR assays, using a nuclear-localized U6 RNA and a cytoplasm-localized 7S RNA as the controls (Fig. 1A).

3.2. Interaction between MALAT-1 and hnRNP C

Long non-coding RNAs are transcripts with little or no protein-coding potential [19]. Multiple studies have reported that they may function through interacting with protein factors [10,20–22]. To explore the biological importance of the nuclear-cytoplasmic translocation of MALAT-1, RNA pull-down assay was first carried out to characterize potential MALAT-1-interacting proteins during the process. To this end, biotin-labeled and unlabeled fragments of MALAT-1 RNA were synthesized by in vitro transcription and incubated with cell extracts, so as to conjugate with potential MALAT-1-interacting proteins. The RNA–protein complexes were then isolated using streptavidin-coated Sepharose, and resolved in an SDS–PAGE gel (Fig. 2A). A differentially presented protein band was isolated from the biotin-labeled RNA–protein complex, and its protein components were characterized by mass spectrometry assay (MS). In total, 18 independent proteins were identified (Supplementary Table 1) and six of them have RNA-binding domains (Table 1). Among these, hnRNP C protein was of particular interest due to its RNA-binding capability and the reported cytoplasmic translocation in the G2/M phase.

To demonstrate the interaction between hnRNP C and MALAT-1, a systematic RNA pull-down assay was performed. Using multiple biotinylated RNA fragments covering the whole MALAT-1 transcript, interacting-proteins were isolated and among them, the presence of hnRNP C was demonstrated by Western blot (Fig. 2B). Compared to the insufficient binding capacity at the 5′ end of the transcript, strong binding to hnRNP C was shown for the other parts of MALAT-1.

To further characterize the in vivo interaction between MALAT-1 and hnRNP C, cell-based RNA immunoprecipitation (RIP) assays were carried out in HeLa cells. Using anti-hnRNP C antibody, hnRNP C-conjugated RNAs were isolated and among them, the presence of MALAT-1 was determined by gene-specific RT-PCR (Fig. 2C). Taken together, these lines of evidence demonstrated a physiological interaction between MALAT-1 and hnRNP C.

3.3. Silencing MALAT-1 expression compromised the translocation of hnRNP C

Previous studies indicated that hnRNP C protein translocated from the nucleus to the cytoplasm in G2/M phase, enhancing IRES-mediated translation and leading to mitosis regulation [13,15]. To confirm these observations in our system, cytoplasmic and nuclear proteins were isolated from synchronized and un-synchronized HeLa cells, the distribution of hnRNP C protein was then investigated by Western blot. In agreement with earlier studies [13,15], our results showed that hnRNP C partially translocated from the nucleus to the cytoplasm in G2/M-phase-synchronized cells (Fig. 3A). Compared to unsynchronized cell populations, a significant increase of cytoplasmic hnRNP C level was identified. This is in sharp contrast to the distribution of Lamin A/C and GAPDH, which are known localized predominately in the nucleus or the cytoplasm (Fig. 3A). To further demonstrate the MALAT-1-hnRNP

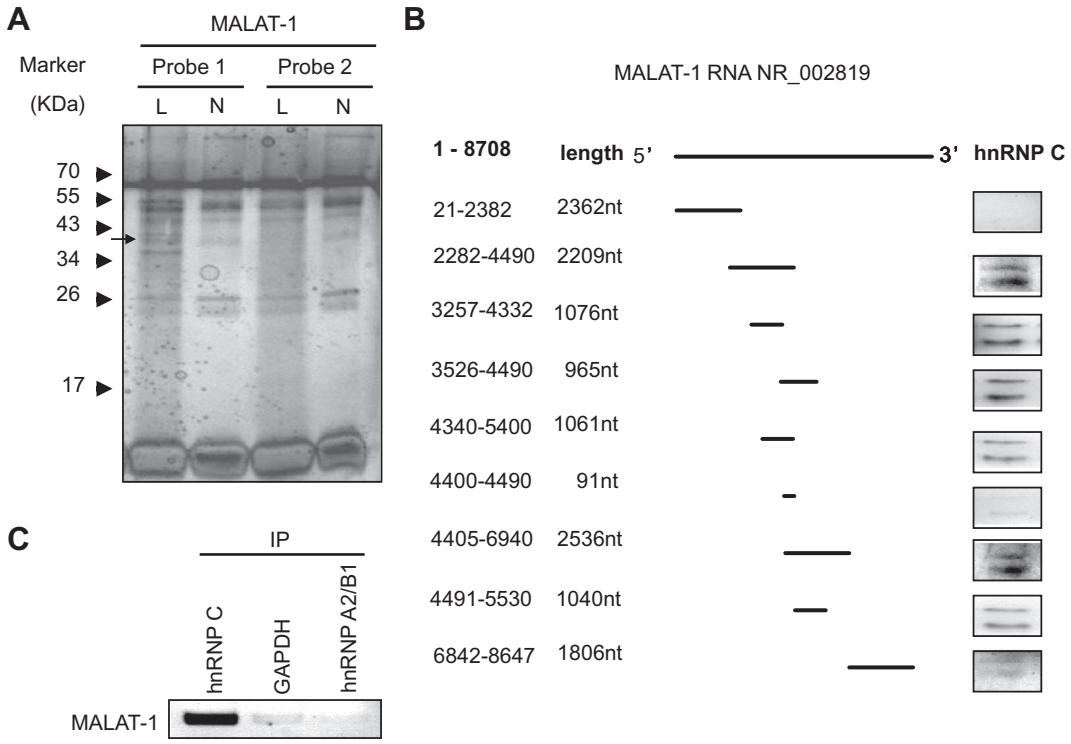

Fig. 2. Interaction of MALAT-1 and hnRNP C in vitro and in vivo. (A) Identification of MALAT-1-interacting proteins. Biotin-labeled MALAT-1 probes are used to isolate MALAT-1-interacting proteins, which are then resolved in SDS–PAGE and visualized by silver staining. A differentially presented band indicated by arrow is picked for component proteins identification by nanoLC-LTQ-Orbitrap-MS/MS assay. MALAT-1 probe 1, position 2282–4490 nt along MALAT-1 transcript; MALAT-1 probe 2, position 21–2382 nt along the transcript. L, biotin-labeled probe; N, unlabeled probe. (B) Characterization of the hnRNP C-interacting regions in MALAT-1 transcript. RNA fragments corresponding to different regions in MALAT-1 transcript are labeled by biotin using in vitro transcription and used to isolate MALAT-1-interacting proteins; the presence of hnRNP C is then assessed by Western blot using anti-hnRNP C antibody. (C) in vivo interaction of endogenous MALAT-1 and hnRNP C. RNA immunoprecipitation (RIP) is performed with HepG2 cell extracts to enrich RNAs interacting with hnRNP C, GAPDH or hnRNP A2/B1 proteins, the presence of MALAT-1 RNA was determined by MALAT-1 specific RT-PCR.

Table 1
MALAT-1-interacting proteins identified by nanoLC-LTQ-Orbitrap-MS/MS.

Gene symbol	Mass (Da)	Gene name	RNA binding domain	Function
HNRNPC	34	Heterogeneous nuclear ribonucleoprotein C1/C2	Yes	mRNA splicing and processing
HNRNPF	46	Heterogeneous nuclear ribonucleoprotein F	Yes	Alternative splicing
HNRNPH1	49	Heterogeneous nuclear ribonucleoprotein H1	Yes	Alternative pre-mRNA splicing
HNRNPH2	49	Heterogeneous nuclear ribonucleoprotein H2	Yes	Thymidine phosphorylase (TP) splicing
HNRNPUL1	96	Heterogeneous nuclear ribonucleoprotein U-like 1	Yes	DNA damage; nucleocytoplasmic RNA transport
SS-B/La protein	47	Sjogren syndrome antigen B	Yes	Diverse aspects of RNA metabolism; enhancing translation

C interaction in G2/M phase, an RIP assay was perform with G2/M-phase-synchronized cells using anti-hnRNP C antibody. Among the isolated hnRNP C-interacting RNAs, the presence of MALAT-1 transcripts was validated by gene-specific RT-PCR, therefore demonstrating the interaction of cytoplasmic MALAT-1 with hnRNP C in G2/M-synchronized cells (Supplementary Fig. 2A). Anti-hnRNP A2/B1 and anti-IgG antibodies were included in the assay as controls, and showed no discernible MALAT-1 signal (Supplementary Fig. 2A).

Taken these evidence together, we hypothesize that the demonstrated interaction, as well as the cytoplasmic translocation of MALAT-1 and hnRNP C, might be synergistic. To test this possibility, siRNAs target MALAT-1 and hnRNP C were designed and their gene silencing activities were assessed using qRT-PCR (Supplementary Fig. 2B and C). By silencing the expression of MALAT-1, we found that less hnRNP C protein translocated to the cytoplasm in G2/M phase, in comparison to the irrelevant siRNA controls (Fig. 3B). Accordingly, enrichment of hnRNP C was observed in the nucleus, compared to the control. Western blot performed with total proteins however showed that the total amount of hnRNP C protein was unchanged (Supplementary Fig. 2D), indicating that silencing MALAT-1 expression compromised only the cytoplasmic translocation but not the expression of hnRNP C.

3.4. MALAT-1 interacts with hnRNP C in cell cycle regulation

The co-translocation of MALAT-1 and hnRNP C in the G2/M phase led us to speculate that MALAT-1 may play an essential role in cell cycle regulation by interacting with hnRNP C. To test this hypothesis, siRNAs targeting MALAT-1 or hnRNP C were individually transfected into HeLa cells, and cell cycle profiles were determined by PI staining and flow cytometry assay, 48 h after cell transfection. Compared to the control, down-regulation of hnRNP C expression led to accumulation of the cells in G2/M phase (Fig. 3C), which is consistent with previous reports [15]. Our study further showed that down-regulating the MALAT-1 level also resulted in enhanced accumulation in G2/M (Fig. 3C). More interestingly, the gene-silencing activity of these siRNAs correlated very

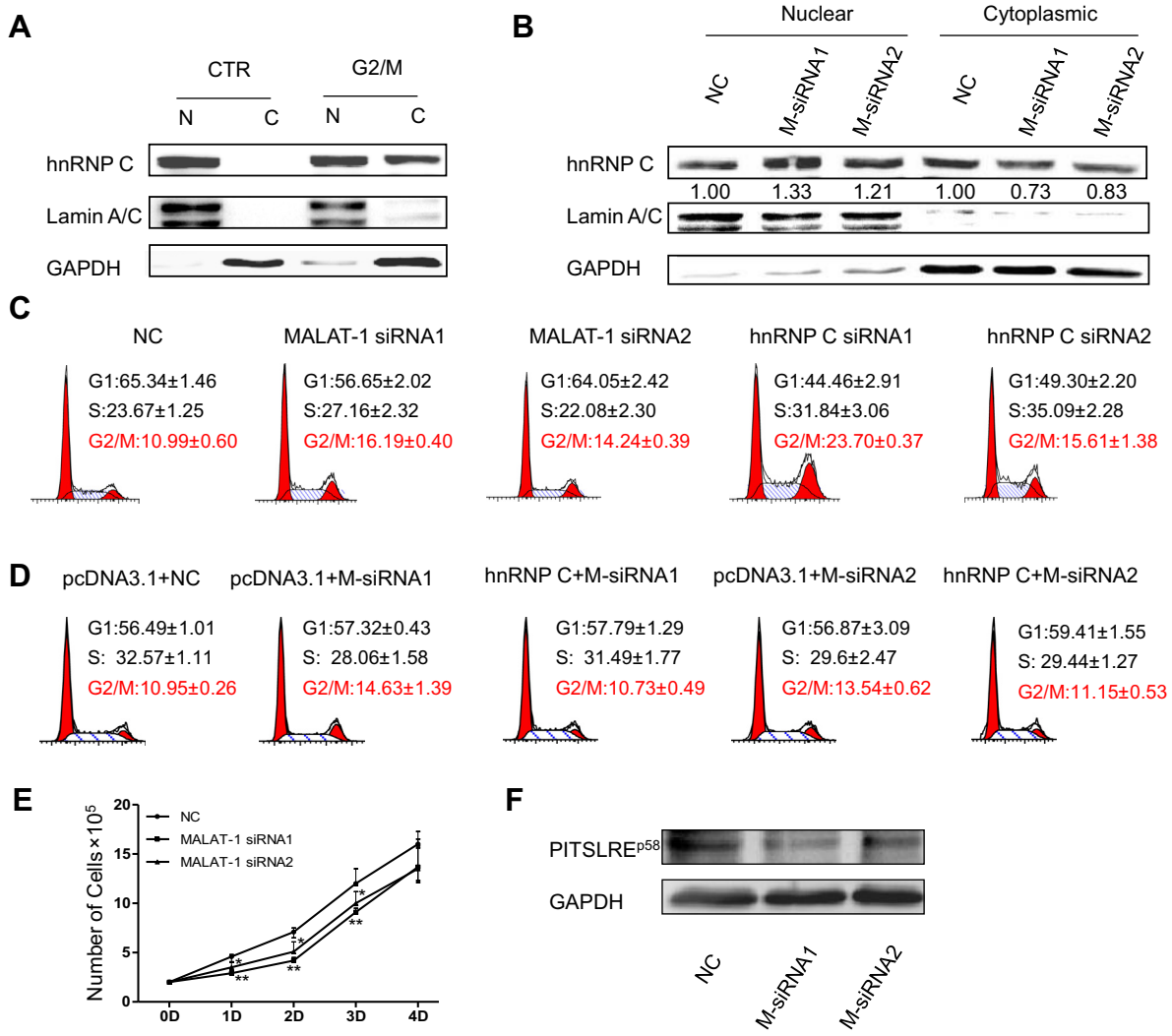

Fig. 3. Silencing the expression of MALAT-1 leads to compromised translocation of hnRNP C and G2/M arrest. (A) Translocation of hnRNP C from nucleus to cytoplasm in G2/M phase. Cytoplasmic and nuclear proteins are isolated from unsynchronized (CTR) and synchronized (G2/M) cell populations. The levels of hnRNP C are determined by Western blot using anti-hnRNP C1/C2 antibody. Lamin A/C and GAPDH are included as nucleus and cytoplasm marker proteins. N, nuclear proteins; C, cytoplasmic proteins. (B) Silencing of MALAT-1 compromises the translocation of hnRNP C in G2/M phase. Nuclear proteins and cytoplasmic proteins are isolated from synchronized HeLa cells, in which the expression of MALAT-1 is knocked-down by individual transfection of two siRNAs. The levels of hnRNP C are determined by Western blot using anti-hnRNP C1/C2 antibody, and further quantified using ImageJ software. Mean values from three independent assays are presented. (C) Silencing the expression of MALAT-1 or hnRNP C leads to G2/M arrest in HeLa cells. HeLa cells are transfected with MALAT-1-targeting or hnRNP C-targeting siRNAs before the cell cycle profiles are determined by propidium iodide (PI) staining and flow cytometry. The percentages of cell populations in each cell cycle phase are determined using ModFit software. The data is presented as mean ± S.E.M. from at least three independent experiments. NC, cells transfected with an irrelevant siRNA; MALAT-1 siRNA1, cells transfected with MALAT-1-targeting siRNA1; MALAT-1 siRNA2, cells transfected with MALAT-1-targeting siRNA2; hnRNP C siRNA1, cells transfected with hnRNP C-targeting siRNA1; hnRNP C siRNA2, cells transfected with hnRNP C-targeting siRNA2. (D) Over-expression of hnRNP C rescues the HeLa cells from G2/M arrest. In which, Hela cells are transfected with MALAT-1-targeting siRNA together with hnRNP C over-expression vector, or pcDNA3.1 control vector. The effects to cell cycle profile were then determined by flow cytometry assay. pcDNA3.1+NC, cells transfected with pcDNA3.1 control vector and an irrelevant siRNA; pcDNA3.1+M-siRNA1, cells transfected with pcDNA3.1 control vector and MALAT-1-targeting siRNA1; hnRNP C+M-siRNA1, cells transfected with hnRNP C over-expression vector and MALAT-1-targeting siRNA1; pcDNA3.1+M-siRNA2, cells transfected with pcDNA3.1 control vector and MALAT-1-targeting siRNA2; hnRNP C+M-siRNA2, cells transfected with hnRNP C over-expression vector and MALAT-1-targeting siRNA2. (E) Cell proliferation assays. $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$. (F) Silencing of MALAT-1 reduced the expression of PITSLRE^{p58} protein in G2/M phase.

well with their function in G2/M arrest. To further corroborate these findings, rescue experiments were carried out by co-transfection of MALAT-1-targeting siRNA and hnRNP C over-expression vector into Hela cells (Fig. 3D). Cell cycle profiling indicated that over-expression of hnRNP C rescues the cells from G2/M arrest, in comparison to the control assays. When Hela cells were treated with MALAT-1 targeting siRNAs, their proliferation was significantly compromised (Fig. 3E). Down-regulation of MALAT-1 also reduced the expression of PITSLRE^{p58} gene (Fig. 3F), which is in agreement with previous studies [15,23]. Taken together with earlier data showing that down-regulation of MALAT-1 compromised the cytoplasmic translocation of hnRNP C in G2/M phase, these lines of evidence demonstrated that long non-coding MALAT-1 functions in cell cycle regulation by facilitating the cytoplasmic translocation of hnRNP C protein.

Based on these findings, a functional model was proposed to integrate long non-coding RNA MALAT-1 into the established protein network in cell cycle regulation (Fig. 4). With this model, long non-coding RNA interacts with protein factor hnRNP C in the nucleus in interphase and when the cells progress into G2/M phase, MALAT-1 facilitates the translocation of hnRNP C from the nucleus to the cytoplasm, stimulates IRES-dependent translation, and increases the levels of CDK11/PITSLRE^{p58} protein in mitosis. The CDK11/PITSLRE^{p58} protein then functions in essential cell cycle

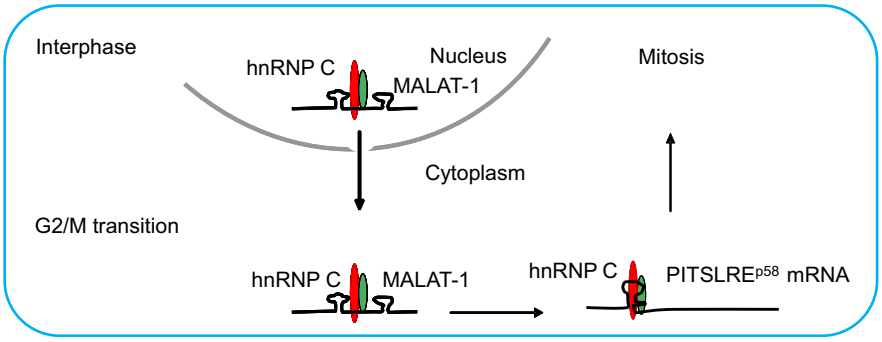

Fig. 4. Association model of MALAT-1 and hnRNP C and its implications for cell cycle regulation. In interphase, MALAT-1 is associated with hnRNP C in the nucleus; and in the G2/M phase, the MALAT-1-hnRNP C complex translocates to the cytoplasm, where hnRNP C regulates PITSLRE^{p58} IRES-mediated translation. PITSLRE^{p58} is essential for centrosome maturation and bipolar mitotic spindle formation during mitosis.

regulation activity, including centrosome maturation and bipolar mitotic spindle formation during mitosis phase [15,23]. When the expression of MALAT-1 was knocked down, the cytoplasmic translocation of hnRNP C was compromised, which may further led to decreased CDK11/PITSLRE^{p58} protein levels and G2/M arrest.

4. Discussion

MALAT-1 is an abundant long non-coding RNA enriched in the nucleus, and it functions in many biological processes [24,25]. Using loss-of-function assays, studies showed that down-regulation of its expression led to cell arrest in the G1/S or G2/M phase, indicating that MALAT-1 may play an essential role in cell cycle regulation [9,10]. In contrast to these in vitro studies, in vivo studies revealed that MALAT-1 depletion did not change the splicing factors level and the phosphorylation status, or pre-mRNA splicing pattern [26–28] in MALAT-1-KO mice, likely suggesting that MALAT-1 is not an essential factor in mouse development. However, more recent study found that depletion of MALAT-1 in cancer cells did impaired its migration and led to less tumor nodules in a mouse xenograft model [29].

Long non-coding RNAs function in many biological contexts and most were found through their direct interactions with protein factors or their guidance of long non-coding RNA–protein complexes to their target sites to regulate gene expression [20]. MALAT-1 is an 8-knt-long macromolecule that can form a complex structure to recruit many different protein factors for functionality. In this study, we showed that MALAT-1 interacted with hnRNP C and facilitated its cytoplasmic translocation in the G2/M phase, thereby regulating the progress of the cell cycle. The distinct cell cycle-dependent translocation of MALAT-1 found in the study adds another layer of complexity and diversity to the functional mechanisms of long non-coding RNAs.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.febslet.2013.07.048.

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